GENETIC ASPECTS OF VIRULENCE IN BACTERIA AND VIRUSES[1]

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At Iowa State College we consider that disease is due to the interaction of four major variables: the genetic constitution of the host for disease susceptibility or resistance, the genetic constitution of the pathogen for virulence or avirulence, the dose of the pathogen to which the host is exposed, and a multitude of variables which we ordinarily include in any genetic experiment under the head of environmental effects. For disease to be produced, the genetic constitution of the host must be a mirror image of that of the pathogen in that a genetic constitution for susceptibility has a relatively low survival value against all organisms whether they are virulent or avirulent. A host constitution for medium susceptibility has a fairly high resistance against avirulent organisms, medium resistance against medium virulent organisms, and a small resistance to the highly virulent type. A highly resistant host has high resistance for all organisms except the most virulent to which they now and then succumb.

HOST MATERIAL

The studies herein reviewed were started in 1925 by differentiating a single host strain for mice and for the domestic fowl into forms highly resistant to *Salmonella typhimurium* and *Shigella gallinarum* respectively. From earlier experiments a dose of 5×10^4 organisms per mouse was chosen as the agent by which resistant strains were established from the previously highly susceptible strains. Similarly for poultry a dose of 1.2×10^7 of the fowl typhoid organism *Shigella gallinarum* was chosen. These organisms were inoculated intraperitoneally. Animals which survived in the best condition in each generation were used as the parents for the next generation. The results of the first fourteen successive generations of selection are shown in fig. 1. Intense inbreeding was used for each group to purify the genetic constitution.

The graphs of fig. 1 show that for both hosts the resistance increased rapidly at first, then somewhat more slowly for six or seven generations, the ultimate survival value of each group being 80 to 90 per cent. In the eighth generation for the mice the dosage of organisms was increased to 2×10^5. This increase was accompanied by a 10 per cent reduction in survival. From that point on the resistance increased again, 93–96 per cent resistant animals being reached in the 14th generation. The results show that despite continuous selection and inbreeding for eight generations there was further residual variation within the strain. Chicks of the eleventh generation were not tested. Subsequent tests showed high

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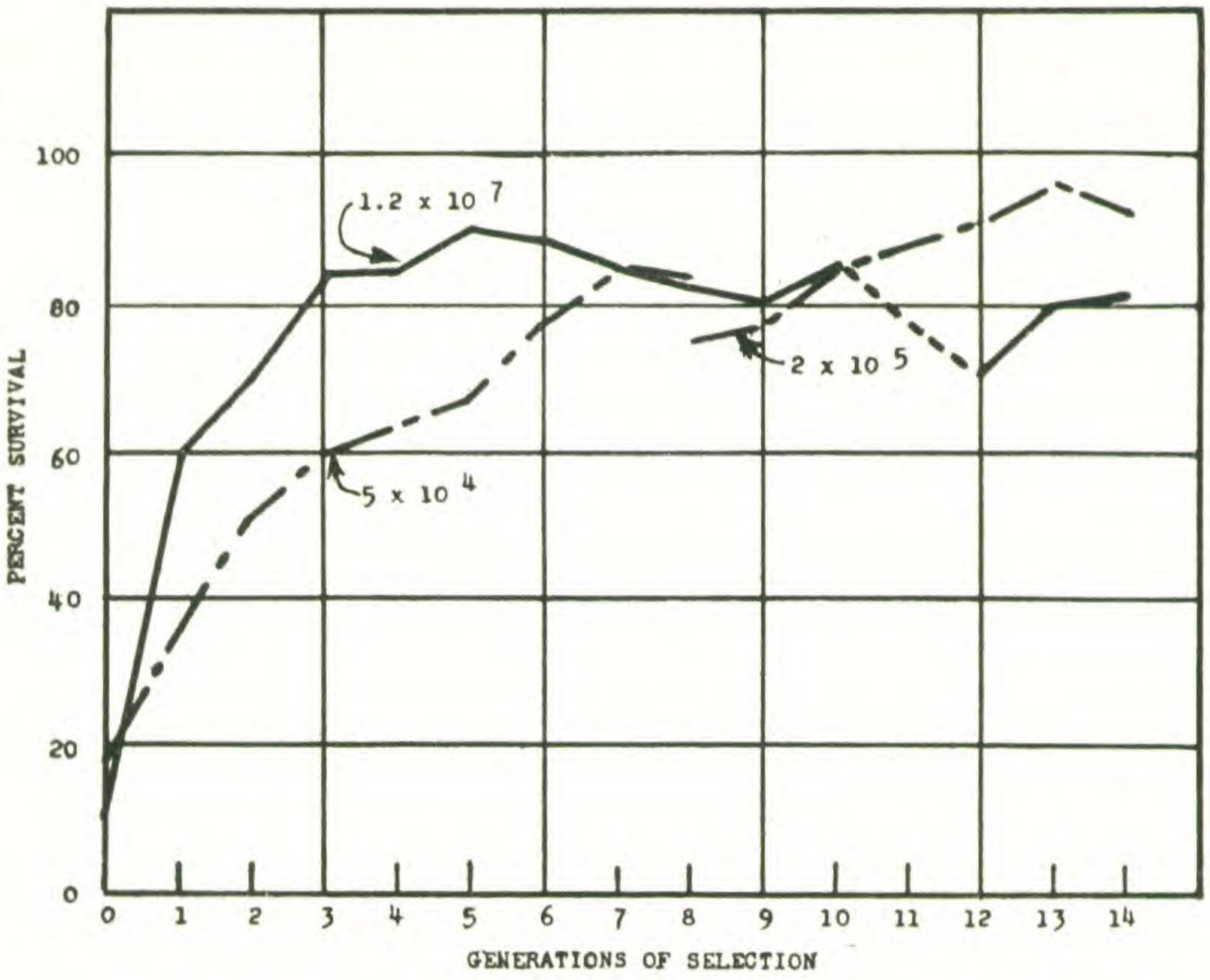

Fig. 1. Survival value of mice (Schott, '31, '32 and Hetzer, '35, '37) and the domestic fowl (Lambert, '31, '36) for successive generations of selection toward the resistant types. Solid line for fowl, dot-and-dash line for mice. Eleventh generation of fowl selection was untested. Eighth generation of mice had dosage changed from 5×10^4 to 2×10^5.

resistance within the chickens. Testing of the parents is not necessary for the resistance in the progeny. The chickens and mice have kept their high genetic resistance despite the fact that the inbreeding has led to the accumulation of genes for smaller size, lower fertility, and some apparent lack of vigor.

Two possible explanations might account for this change in resistance: selecting of small variations due to genes for resistance already in the parental lines or selecting of mutations toward higher resistance, each mutation being partly dependent on the total genetic constitution of the host for disease resistance. Either explanation would lead to the disease resistance observed in these lines. Actually both factors appear important. There is some experimental evidence to indicate that if a sufficiently large population of mice is chosen it is possible to pick out from this population individuals which carry very high genetic resistance to mouse typhoid and actually make the change from a relatively susceptible population to a highly resistant population in a very few generations. This sudden change could favor the view that disease resistance may be accomplished in one step or may be due to a single gene pair. The results of Hetzer show that this is not the case. The circumstances leading to the choice of resistant mice are fortuitous, a result of a combination of several genes for resistance in one animal brought about by chance segregation.

A second question of interest is why a completely resistant strain is not attained. The strains which have been formed would be considered completely resistant if bacteria of low virulence were used to initiate the disease. With highly virulent organisms, some deaths do occur. It seems that no species of animals having a native disease has yet had a completely immune race established through genetic means or any other means for that matter. Highly resistant animals have been produced but with highly virulent organisms of the pathogen it is possible to produce some deaths in all cases.

Since the fourteenth generation the selected strains have been maintained without testing. The resistance of the present generation is as high as it was under testing. Genetic resistance when made homozygous for the strain is a permanent attribute of the strain.

PATHOGEN VARIATION

In November, 1940, our culture of *Shigella gallinarum* was found to have completely lost its virulence when used in a fairly large test. This culture had been highly virulent the previous May. The results demonstrate that a large population of virulent bacteria could be replaced by avirulent bacteria in a period of something like seven months. This fact was of particular interest since this line had previously retained its pathogenicity for several years.

The mechanism of such changes was not entirely unknown to us as our previous studies of such genetic variation of the pathogen had given us a fair understanding of how they came about. The following investigators made large contributions to this problem and to the others herein discussed. The results reported are the joint effort of the following workers in our laboratory: Dr. M. R. Zelle, Miss Janice Stadler, Mr. G. W. Kohler, Mr. John A. Weir, Mr. A. E. Bell, Mr. E. F. Oakberg, and Dr. R. E. Lincoln.

The avirulent culture was subjected to the proper cultural and serological studies to prove that it was *Shigella gallinarum*. The culture was then inoculated into a chicken of a very susceptible strain. Five different isolations were made from this host. Two of these isolations did not progress very far, one being lost in the first passage and the other one shortly thereafter, indications of the low virulence of the *Shigella* culture.

Isolations were made from the heart, liver and spleen respectively. As no small chicks were available, these lines of bacteria were passed through six successive 10-week-old birds of the susceptible strain, each line being kept separate from the others.

The bacteria were kept in the chickens one week, then for three days on culture media at each passage. The inoculating dose was two billion organisms. These passage birds showed no mortality, but the organism was recovered from each bird inoculated. Tests for virulence on the seventh passage organisms were made on 10-day-old chicks. One line, D7, killed 10 out of 10 chicks in less than 10 days. The second line killed 6 of 11 chicks but took 21 days to do it. The third line

killed 8 of 11 chicks but also took 21 days to do it. A transfer culture of the parent avirulent culture from which the above lines originated killed 3 of 11 chicks in 21 days. It is evident that one of these strains, D7, differs from the others in virulence.

Further analysis of line C showed that for an average dose of 5×10^6 it killed only 6 out of 35 resistant chicks and 11 out of 37 susceptible chicks. Line D killed 13 out of 41 resistant chicks and 36 out of 36 susceptible chicks. Line E killed 11 out of 40 resistant chicks and 34 out of 34 susceptible chicks. The percentage comparisons were for the resistant line 17, 32, 27, and for the susceptible chicks 30, 100 and 100, for lines C, D and E respectively. The parent avirulent culture showed 24 per cent mortality in the susceptible host. Two relatively pathogenic lines had been established from a highly avirulent line. The mechanism of this selection is important.

To determine the variability of *Shigella gallinarum* under natural conditions, a survey was made of cultures from chickens diagnosed as clinical fowl typhoid during the summer. Sixteen cultures and 11 sub lines showed marked variability in the end point for agglutination in anti *Shigella gallinarum* and/or anti *Salmonella typhimurium* serum, metabolism of sugars, colony morphology and pathogenicity. The species *Shigella gallinarum* evidently had wide genetic variability.

ANALYSIS OF VIRULENCE CHANGES

Experiments were planned to analyze bacterial variability as it is related to the genetics of virulence. From the avirulent stock culture described above 20 colony isolations were made. As this organism does not clump appreciably, each of these colony isolations probably represents the descendants of a single bacterium. Ten of these avirulent lines were exposed to the environment of our inbred, highly resistant chickens described above. These inbred lines are capable of surviving nearly 1000 times the number of bacteria which will cause death in most flocks. The other ten strains of avirulent bacteria were grown in a strain of chickens marked by susceptibility to fowl typhoid. Two chicks were used at each passage for the resistant host line and one chick for the susceptible host line. The avirulent strains of *Shigella gallinarum* were thus exposed, on the one hand, to the intensely unfavorable environment of the resistant strain of host, and, on the other hand, to the more favorable environment of the highly susceptible host.

Attempts were made to pass each culture successively through 16 different 10-day-old chicks using the technique described above. Despite the fact that twice as many chicks were available for recovering the organism at each resistance passage, 24 passages were lost in the transfers through the resistant host compared to 10 for the susceptible series. Life for the typhoid bacteria in the resistant host was tough. The avirulent strain has great difficulty in establishing itself even to making a mild disease in the resistant host strain. This fact suggests that the resistant host would be a potent selecting force tending to pick out the progeny

of any variants characterized by increased virulence.

Small tests were made throughout the passage experiments to determine the constancy with which the organisms recovered retained their virulence. A larger test was made at the end of the experiment to establish more exactly the virulence of each line. The rather scattered and low amount of data taken during the passage of the 20 lines of bacteria through their respective hosts show that each strain retained its low virulence for a varying number of passages. Changes when they did occur came suddenly during a single passage and resulted in a substantial gain in virulence, the total amount of change differing for different strains. When a change in virulence did occur, the subsequent tests showed a retention of the new virulence. These results favor mutation and subsequent replacing of the avirulent type by the virulent mutant.

Tests of the 20 lines at the end of the sixteenth passage give further support of this conclusion. Two of the lines, I and R, had not changed in virulence as the result of growing in their natural host for half a year (fig. 2). One line was carried in the resistant host. The other line was passed through the susceptible host. If virulence is due to chance mutation, the expectation would be essentially equal numbers of mutations in each group. The observations bear out this hypothesis. Two lines of medium virulence have been established from the resistant host against three lines for the susceptible host. Seven highly virulent lines came from resistant host passage and six from susceptible host passage.

The over-all picture for the 10 lines passed through the resistant hosts was as follows: A dose of 100,000 organisms inoculated into 74 resistant chickens led to 20 per cent death; inoculated into 203 susceptible chickens led to 70 per cent death. With 100,000,000 organisms as the dose, 70 resistant chickens had 22 per cent death, 124 susceptible chickens had 88 per cent death. For the lines derived by passage through the susceptible host the 29 resistant chickens with 100,000 dosage had 7 per cent death and the 186 susceptible chickens had 84 per cent death. For the 100,000,000 dosage 54 resistant chickens had 22 per cent death, and 98 susceptible chickens had 86 per cent death. These data show that passage through either host is equally favorable to establishing of virulence. The degree of increase in virulence may be judged by the fact that the original avirulent culture inoculated in 100,000,000 organisms showed no death on the resistant host and only 34 per cent on the susceptible host.

The chicks of either strain are highly efficient selective agents favoring any variants toward virulence and encouraging them to multiply at the expense of the avirulent type. The population within the host becomes rapidly purified towards the virulent type. The genetic constitution of the domestic fowl, the natural host to this disease, is sufficient to create the necessary conditions for this selection process. The culture media, on the other hand, appears to favor those organisms whose genetic constitution is for a saprophytic type of growth.

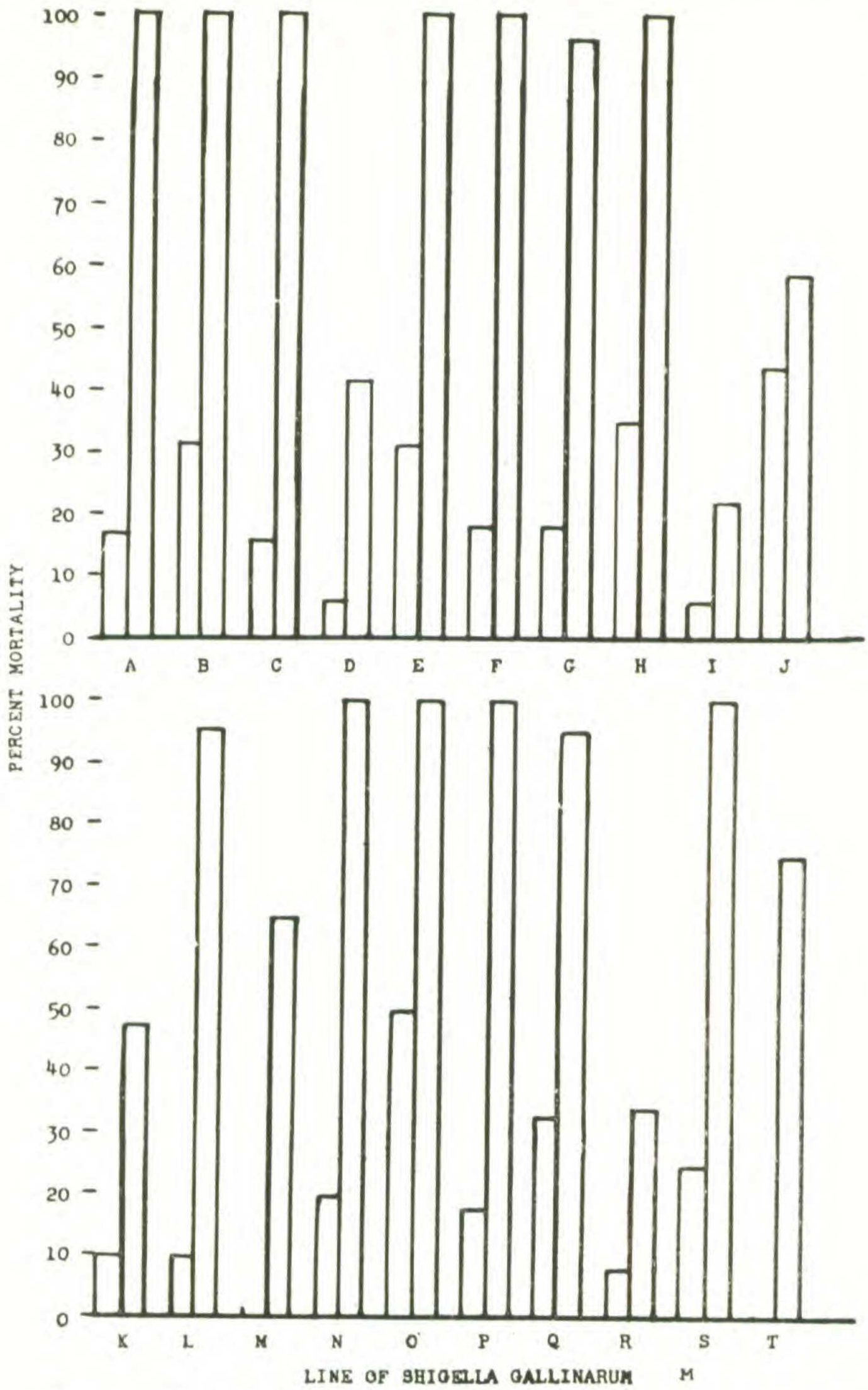

Fig. 2. Virulence of different lines of *Shigella gallinarum*, originating from the same avirulent line, after 16 passages through resistant chicks A to J and susceptible chicks K to T. Left, ordinate tests on resistant chicks; right, ordinate tests on susceptible chicks.

GENETICS OF VIRULENCE IN MOUSE TYPHOID

Experiments of a similar nature were carried on earlier in this laboratory utilizing *Salmonella typhimurium*, the agent of mouse typhoid. A single laboratory line of *Salmonella typhimurium* was available. This line had retained constant virulence on culture media for more than ten years. Six different experiments were performed, each varying somewhat, but all directed toward detecting and tracing virulence changes in this line. Six different strains of mice, differing in their resistance to mouse typhoid, were available.

In some experiments the bacteria selected for use were the direct descendants of a single organism picked out by the micropipette. In others, the organisms were the result of five successive platings and single colony isolations. The initial bacterial line chosen had essentially the same virulence as the parent culture. The parent culture was different from that described for the domestic fowl in that it was originally a culture of medium virulence.

The culture was divided into two parts: one part exposed to the effects of the environment of the resistant host strains of mice, the other part to the environment of susceptible host strains of mice. The longest experiment performed involved 36 successive passages of bacterial line from one mouse to another and covered a period of two years. The outcome of these experiments brought out several facts important to our interpretation of the physical basis for virulence of a disease-producing organism.

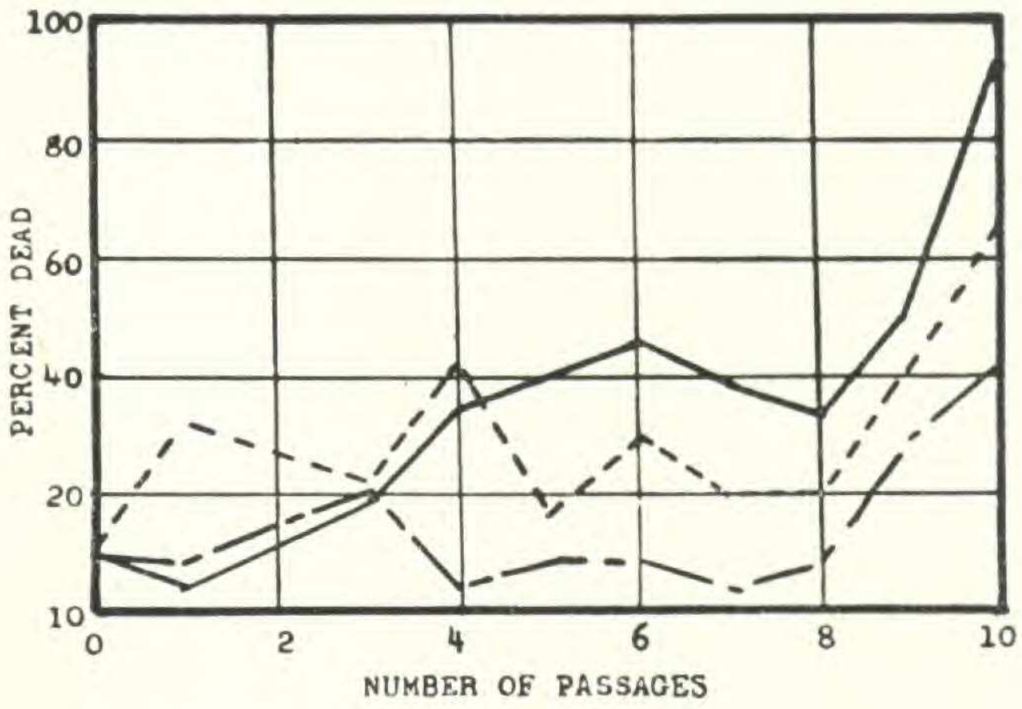

Fig. 3. Changes in virulence observed in the passage of a medium-virulent line through susceptible mice, through resistant mice, and as kept on culture media. Solid line is for the passage through resistant mice; dotted line, for passage through susceptible mice; and dash-dot line, for the culture on culture media. Tests made on resistant mice.

In general, the stability of our original pathogenic line was demonstrated. Increased virulence, when it was observed, occurred only in a low proportion of cases following passage of recently isolated single-celled cultures. Changes in virulence, when they did occur, were sudden. The increased virulence observed was then subsequently maintained at the new high level. There was no suggestion of a gradual accumulative effect of the environment on these increases in virulence. Lines of the pathogen more virulent than the parent culture were obtained in passages through both resistant and susceptible hosts. If virulence increased during passage, the increase was abrupt. Continued growth in the particular host environment resulted in no further increase in virulence. Figure 3 shows one of these experiments.

As bacterial lines were isolated they were sometimes marked by morphological characters along with virulence differences. This association suggests that the phenotypic character of the bacterial colony may be an expression of its virulence.

The correlation is high but not complete. Most but not all of the lines isolated and showing increased virulence went toward the smoother types.

By making use of two bacterial lines differentiated in their colony phenotypes, as well as by their virulence, it was possible to show a very intense selection for the more virulent line of bacteria. Bacteria from the two lines were mixed in definite proportions and then inoculated into mice. At intervals after inoculation bacteria were recovered from the mice, and the proportion of the two types determined. The results showed that the virulent forms quickly became dominant and often were the only type present in the population.

A further confirmation of this fact comes in experiments in which virulent bacteria are inoculated as against those in which avirulent bacteria are used. The bacteria are recovered easily from the host inoculated with virulent culture but with difficulty when inoculated with avirulent organisms.

By growing a large progeny of a single-celled isolation on agar media, it was possible to isolate five different phenotypes appearing as variants of the original culture. Four of these variants showed colonies of greater roughness than the parent. In one the colony was smoother than the parent. This line was also unstable in its phenotype, occasionally producing rough variants. Tests of these phenotypic variants showed that some differed from the parent culture in virulence. The observed facts thus demonstrate the occurrence of phenotypic variations in the progeny of single cells. The amount of this variation is sufficient for the changes in virulence observed in our experiments. Changes in virulence in artificial media are of the same type as those observed in the host. They appear suddenly with sharp differences between the phenotypes.

No relation was observed between the virulence, growth rates, or fermentation reactions of the lines.

In essence the results show changes in virulence to be analogous to mutations in higher forms. The rate of mutation for a given type is small. The changes are sporadic in their appearance and when they do occur are permanent and true-breeding. The variation can go in each direction—toward higher virulence or toward avirulence. The environment of the host or culture medium acts as a selective agent for the genetic type which fits the environment. The environment is not the cause of the variation.

MUTATIONS IN *PHYTOMONAS STEWARTII* AND THEIR RELATION TO VIRULENCE

The problem of virulence and its dependence upon the inherited bacterial constitution may be studied by searching for phenotypical variants in an original pure stock. These variants may occur naturally, under irradiation or in other ways. Two different lines of a corn-wilt organism *Phytomonas stewartii* have been examined for mutations which occurred naturally and after irradiation with X-rays. The first of these lines is a dark yellow rough type with a medium-sized colony. The second type is a large colony with diffuse center. The virulence of these parent lines may be judged by comparing the green weight of plants inocu-

lated with them as contrasted with that of the normal uninoculated plant. Twelve mutants of the dark yellow rough type parent were compared in virulence with the parent type. These mutants were of several kinds. The colonies might be pale yellow, white, roughs of several grades, extreme smooths, mucoid or dry, large or very small. Some of these mutant types, photographed at the same scale and age, are shown in fig. 4.

One parent type had a virulence index of 31, or it was rather low in virulence. Of its 12 progeny mutants 3 were below the parent and 9 were above the parent in virulence. The virulence indexes ranged up to 70. The average was 45. Virulence variations sometimes accompanied the morphological variations and were apparently an expression of the sudden change in type.

Eight variations from the other parent type were selected on the basis of like characters. This parent type had a virulence index of 75. Seven of the 9 mutants tested showed virulence indexes below that of the parent, ranging to as low as 46. Two had indexes above the parent, 81 and 78. The average virulence was 62. The abrupt phenotypic changes in bacterial type observed in this line likewise may affect virulence.

Certain apparent correlations are evident in this comparison. The mutants tend to remain fairly close to the parental type in their virulence. The variation which is observed seems to be directional. When the original parent stock is of rather low virulence, a mutant is most frequently of a somewhat more virulent type. When the parent is of virulent type, then the mutants tend to show less virulence than the parent.

VIRUS MUTATIONS AND PATHOGENICITY

Several investigators working with viruses have noted changes in strain type and in pathogenicity. The analysis of these changes has come particularly in the study of the tobacco mosaic viruses where McKinney noted that suddenly appearing yellow types might be due to mutation from the original form. Jensen isolated over fifty of these variant types occurring normally in ordinary tobacco mosaic. During the course of our own studies on tobacco mosaic large numbers of different variant types have been obtained. These types may be grouped into three major categories: those similar to ordinary tobacco mosaic, those similar to aucuba mosaic, and those producing yellow-mottling rather than the green type. Besides these differences, there are quantitative variations in invasive capacity which seem to be characteristic of the individual variants.

Attempts have been made to determine the inactivation rates of some of these mutants under similar X-ray treatments. The results of these studies indicate that within the limits of accuracy of the X-ray determinations the inactivation rates of the different mutations are the same as those for the parent type. As the different variant types originated from the same parent type, it follows that in so far as this property is concerned, it has been preserved in the variants while they have varied in other directions, i. e., invasive power or phenotypic expression of the host plant. If we take the view that inactivation rates under the same

conditions measure the reproductive size of the virus, it would follow that the size of the virus particle had not changed while the mutation was taking place. The mutation could not be accounted for as splitting of the original particle or as a polymerization to a larger size. Thus the virus may mutate in one characteristic while its other characteristics remain the same as the parent.

Holmes has made a most important study along these lines. He has studied the mutations derived from a masked strain and from a distorting strain of tobacco virus. Thirty-one yellow variants from the distorting strain and 84 variants from the masked strain were observed. Twenty-three out of the 31 variants from the distorting retained fully the systematic invasive power of the parent type. But three of the variant strains produced only local lesions. The mutants from the masked strain, on the other hand, produced no fully invasive types in the 84 which were examined. The changes to the yellow mosaic type in the variants are independent of the invasive characteristic and may represent unit differences in the structure of the viruses similar to such differences in particular genes. These results agree with ours on *Phytomonas* in showing a persistence of virulence type even with marked changes in other characteristics.

These facts gather added significance if the hypothesis that each of these viruses is a unit or molecule is accepted. It would mean that an individual virus may have several side chains or like structures which are capable of affecting the host phenotype in different ways. It will be remembered that tobacco mosaic particles take the form of a long rod composed of smaller units seemingly repeated throughout its length. A single particle could then get its different properties either by different structures of the whole or by having each unit so differentiated as to be responsible for a given reaction.

Each of these different variants of a parent type evidently retains the common characteristics of the capacity for self-reproduction. Added to this basic character, permanent structural alterations may lead to yellow vs. green mottling or localized necrotic lesions vs. the spreading invasive type, etc.

The reproductive capacity is subject to modification by radiant energy and other means. Loss in infectivity may take place under the action of X-rays or ultraviolet light without changes in other properties sufficient to be detected by serological or other means. The presence of these properties is not an indication of the capacity of the virus to reproduce.

Serological techniques of precipitation, complement fixation, or virus neutralization may be used to distinguish different viruses from each other as shown by Chester and others. Strains within a given virus are not readily detected by these means even though these strains show markedly different phenotypic characters in the host plant. These facts indicate that various changes may occur and not be reflected by serological reaction. This may be expected if the particular alteration necessary to produce the new type is not of an antigenic nature. On the other hand, the fact that the larger differences of tobacco mosaic and aucuba mosaic and the enation mosaic may sometimes be detected by serological means indicates that at least some of the changes within these forms are antigenic in nature. One

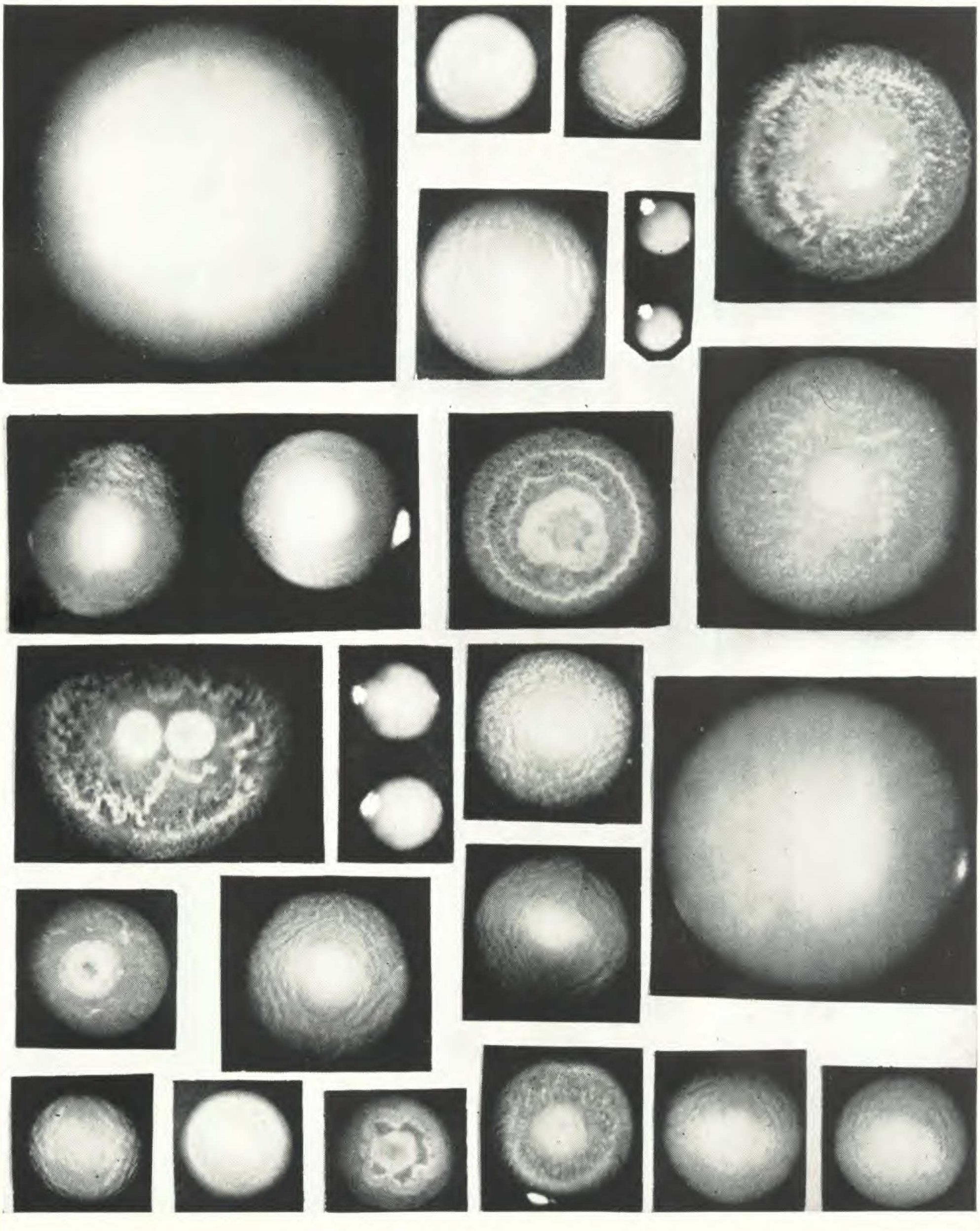

Fig. 4. Mutant types from two lines of *Phytomonas stewartii* differing in virulence.

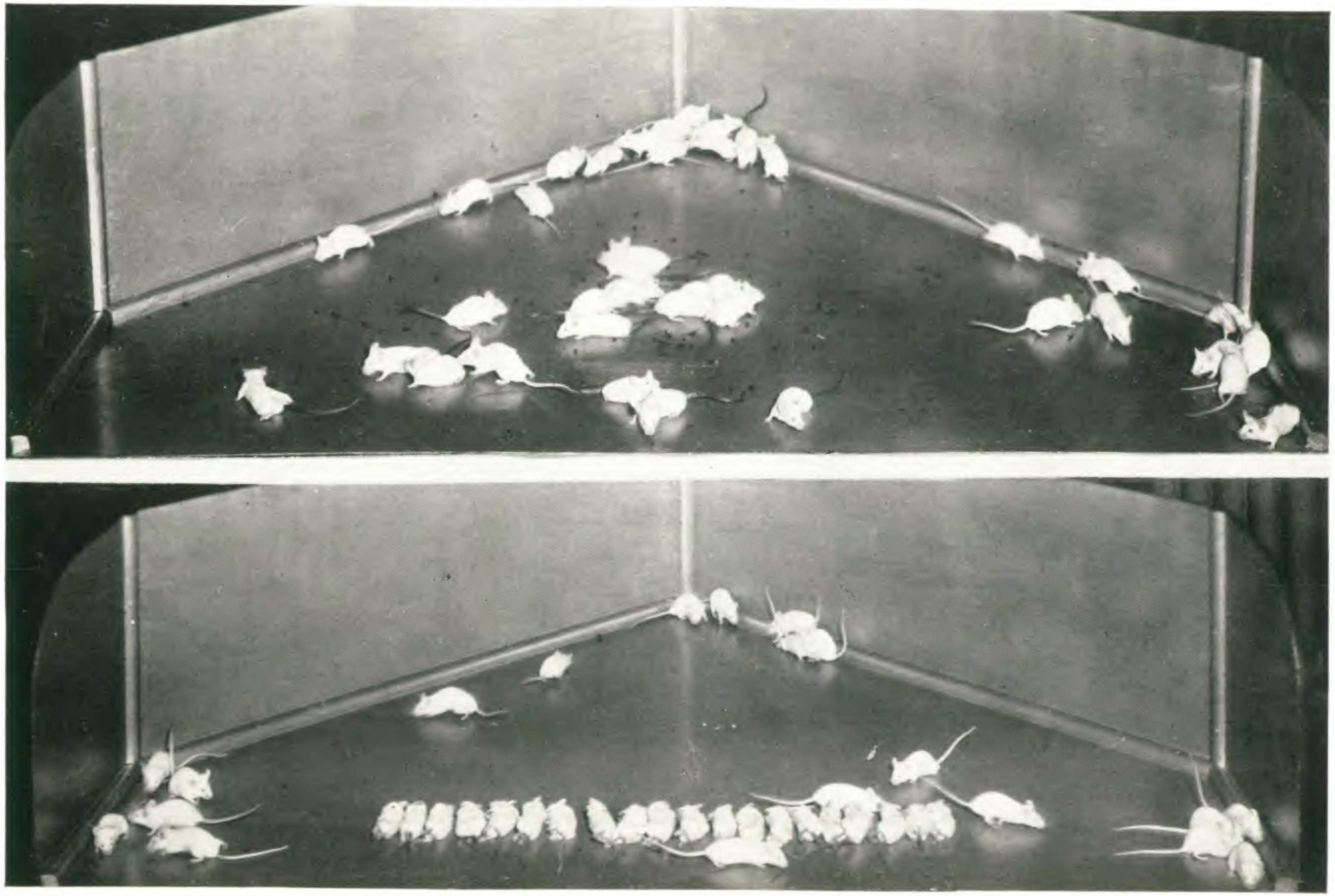

Fig. 5. Photograph showing 20 mice each of the S and Ba strains inoculated with like dose of the same typhoid culture. All the S mice survived; all the Ba mice died.

might assume that such changes involve some type of polysaccharid rearrangement. These facts have a direct bearing on the gene and the detection of its mutants. They suggest that extreme variants of the gene might be detected by a change in its serological structure. Less extreme variants appear much less likely of detection. Under such circumstances the products of the gene's action rather than of the gene's own structural alterations appear more likely of detection.

ORIGIN OF VARIANTS

Variants which occur in cultures *Shigella gallinarum, Salmonella typhimurium* and *Phytomonas stewartii* appear to fall in two distinct categories: those in which the variant is stable from the time of its appearance and those in which the variant breaks up into two types, one of which is stable, the other of which continues to break up into the two types in successive generations. These two categories appear to form a discontinuous series and there is reason to believe that the mechanism behind the changes may be distinct.

The time of bacterial mutation in *Salmonella typhimurium* has been traced under microscopic observation. A smooth mutant type was chosen. This type is called unstable as its colonies are composed of bacteria which, on one hand, give smooth colonies and, on the other, give rough colonies. Bacteria from the rough colonies give nothing but rough. The smooth type will repeat, giving both smooth and rough colony types. The case is therefore comparable to similar unstable mutant types in *Drosophila*.

A single organism of the smooth type was picked up in a micropipette, and placed on a thin agar film under the 4-mm. power of the microscope. This cell divided and the daughter cells were separated from each other with the micro-pipettes. The cells divided again and were again separated. In this manner it was possible to separate and mark the individual cells of six successive divisions. The cells were then allowed to grow into micro-colonies. Each micro-colony was separately picked off, the cells separated by shaking in liquid media and then seeded on agar plates to identify their types. If the colonies were of three types—smooth, mixture of smooth and rough, and rough—the original cell was the smooth unstable type. If the colonies were all rough, the original cell was a mutant to the rough type.

In two such pedigrees a single mutant rough colony was observed, a rate of change of 1 in 134 cells. The sister cell to the rough mutant was smooth. The fact traces the mutations to the events occurring in the division of a single cell into two cells. It shows that the change is not due to any over-all environmental effect for even sister cells do not share the same effects.

The rate of mutation may be checked by a statistical analysis of the relation between the smooth type and rough type found in a single-celled culture after a certain lapse of time where the generation time is known. This statistical analysis will not, of course, substitute for the visual analysis above in showing that the variants occur as one daughter cell of a pair at a single division. Ten separate single-cell isolations were analyzed for their rates of mutation.

The results of this comparison give an average estimate of the mutation rate of 0.0053, or 1 mutation in 187 cells. This agrees rather well with the estimate from the pedigree cultures of 1 in 134 cells.

Observation of a variant appearing as the result of a single cell division has thus been made. The rate of occurrence of these variants is, however, much higher than that observed in the stable variants. An examination of temperature effects on the rate of occurrence of these variants indicates that the mechanism involved for the unstable type as contrasted with the stable may be quite different. In fact, it may be similar to that observed in crossing over or in variegation in *Drosophila*. The results make it highly probable that there are at least two methods by which the variants in bacteria occur.

HOST CONSTITUTION AS RELATED TO BACTERIAL CONSTITUTION IN DISEASE

Three lines of mouse typhoid have been preserved as the result of the above experiments. One is highly virulent; the second is of medium virulence; and the third is nearly avirulent in our customary dosage of 200,000 organisms. The host in which these bacteria have worked have likewise been segregated into strains, the survival value of each particular strain being shown in Table I. Photographs of the outcome of a recent experiment involving 20 mice each of the S and Ba strains strikingly illustrate these differences.

TABLE I

COMPARATIVE RESISTANCE OF Ba, L, E, Z, RI, AND S STRAINS OF MICE TO *SALMONELLA TYPHIMURIUM*, STRAIN 11c, INOCULATED WITH 200,000 ORGANISMS, DATA 1938 TO 1942

Strain	Lived	Total	Survival %
Ba	35	452	8
L	63	470	13
E	292	555	53
Z	733	1262	58
RI	372	496	75
S	988	1150	86

The resistance differences in these strains are genetic. They have been segregated into them and made relatively pure by various means: inbreeding, selection and inbreeding, etc. The strains have held their respective resistance levels for eight or more generations when tested with the same line of mouse typhoid bacteria.

THE CHARACTER BASIS OF VIRULENCE

From the mutations in virulence described above, three lines of the pathogen were preserved. One line is highly virulent, a second has medium virulence, and the third is nearly avirulent in our customary dosage of 200,000 organisms. Hypothetically, virulence in a pathogen could be due to (a) an accentuated capacity for growth to a point where sheer numbers overwhelm the host, or (b) the organism having the capacity to produce a toxin to the host. Some insight into this question was obtained by comparing the lethal action of living and heat-killed cultures (56° C.) of our three bacterial lines. Under these conditions the six strains of mice retained a similar order of resistance to the heat-killed organisms that they had to the live organisms. This indicates that resistance to toxic substances produced by the pathogen is certainly one of the characters involved in genetic resistance or susceptibility. Post mortem examinations, showing that the dead organisms may produce sterile lesions comparable to those observed in the same strains inoculated with the same line of living pathogens, give further support to this view.

However, capacity to grow in the host is also a factor. This is shown by the comparison of the mortalities for the three bacterial lines, when alive or dead. Two of the bacterial lines, differing markedly in live-organism virulence to all mouse strains, showed little difference when compared on a heat-killed basis. Growth rates of the three bacterial lines are equal so that rate of growth of the organism is presumably not a factor. Rather it is the capacity of the organism to grow in the host to numbers which will be lethal. The most lethal bacterial line can grow in mice to the toxic limit. The second pathogen is stopped by the host's resistance before this point is reached. Two genetic characters are thus of demonstrated significance to pathogenic bacteria; the capacity to elaborate toxic products and to multiply rapidly in the host.

CHARACTER BASIS FOR ACTIVE AND PASSIVE IMMUNITY

Mice from the six strains were immunized by inducing clinical typhoid by the live-organism route. The three bacterial lines were used. These mice were subsequently inoculated with a large dose of a single bacterial line. The follow-up dose of live organisms necessary to get a fair death rate is about the same as that necessary to get similar death rates in immunized mice treated with killed organisms. This fact indicates that although antibodies present in the immunized host are able to check the growth of moderate numbers of pathogens, the ability to withstand the toxins produced by the bacterial cells is not greatly altered by the previous immunizations.

But individual host-strain differences appeared on immunization. Using a factorial design some 2700 mice were immunized with killed cultures of three different lines of our bacteria. The mice were equally divided among three different strains, one highly susceptible to typhoid, another of intermediate susceptibility, and a third of great resistance. These mice were immunized once, twice,

or three times. The number of bacteria given at a dose was 1.25×10^7, 1.25×10^6 or 1.25×10^5. The whole design containing 27 treatments was completely balanced, equal numbers of mice being present at each treatment. After immunization and a lapse of 21 days the mice were injected with a rather massive dose of one of the bacterial strains, 5×10^7 organisms of 11 C. The results of these experiments bring out two interesting facts: 1. The three genetically different strains of mice show the same relative resistance to the typhoid organisms after immunization that they had prior to immunization. The dose necessary to bring about death, however, was about a hundred times greater after immunization than before. 2. The bacterial line of low genetic virulence was poor in immunizing capacity. The two virulent lines were both reasonably good immunizers. The results are shown in the succeeding table:

TABLE II

RELATIVE RATES OF SURVIVAL OF IMMUNIZED MICE AFTER INOCULATION WITH 5×10^7 ORGANISMS OF THE VIRULENT CULTURE

Immunizing Organism	Strain of mice			
	Ba	Z	RI	Mean
9 D	8.1	11.0	10.5	9.9
11 C	2.7	37.5	66.3	35.5
DSC1	5.8	30.9	60.5	32.4
Mean	5.6	26.5	45.8	25.9

CHARACTER BASIS FOR INHERITED HOST RESISTANCE

Our studies have shown that genetic resistance to mouse typhoid pathogen can be split up and the host differences segregated into pure breeding strains each characterized by a particular resistance. What inherited characters in the host are responsible for this resistance?

THE BLOOD CELLS

Our first study dealt with the characteristics of the blood cells. These studies showed that numbers of leucocytes were high in the highly resistant lines and low in the susceptible lines, the intermediate lines falling in between these extremes. The correlation between leucocyte numbers and resistance was high. The type of leucocyte did not seem to be of much importance; rather it was the total number. This suggests that the body can call out the type of leucocytes it needs to meet particular environmental circumstances. The numbers of leucocytes are an inherited character of the strains.

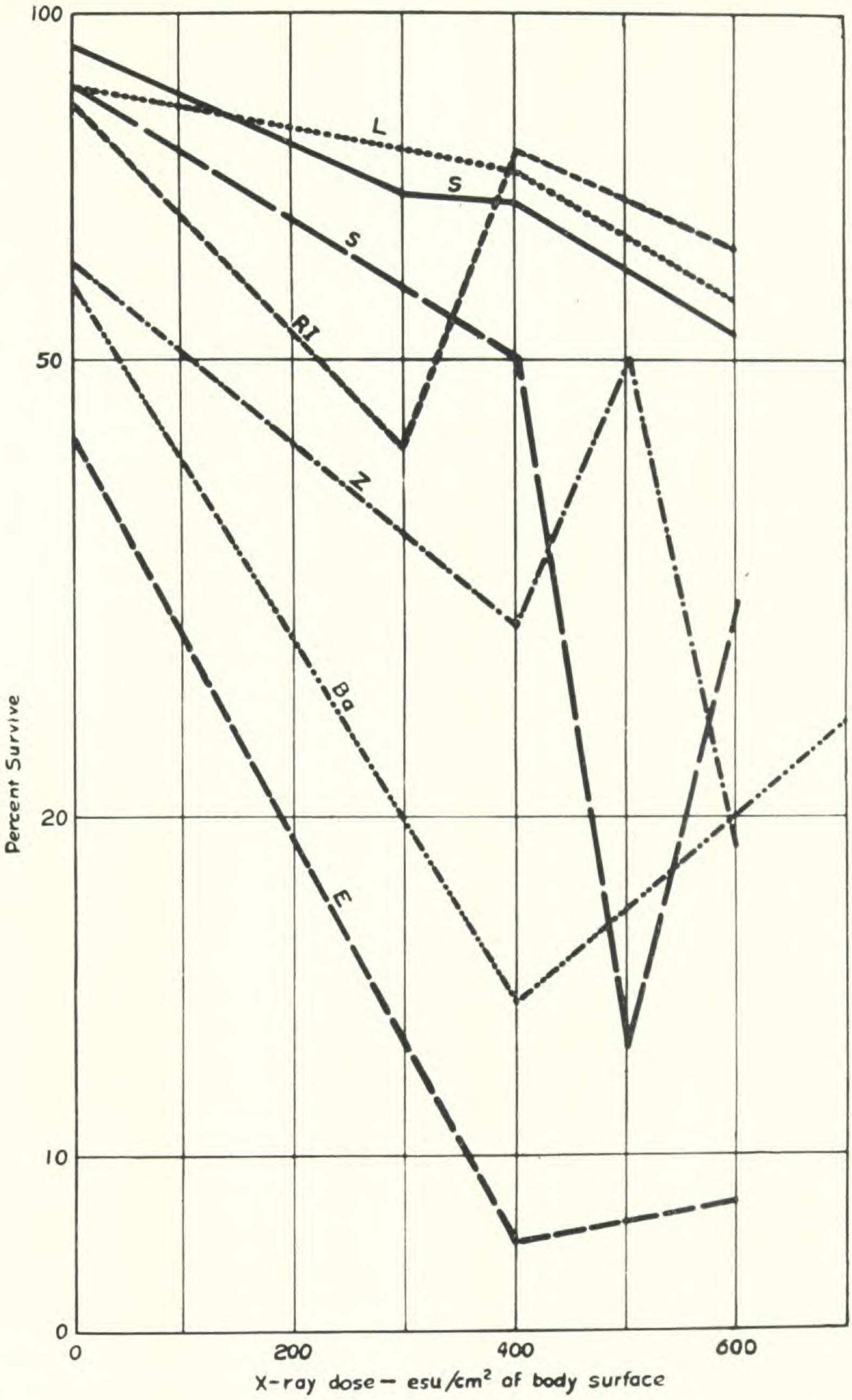

Fig. 6. X-ray treatment of different strains of mice as a means of reducing their resistance to mouse typhoid.

The erythrocyte numbers of the blood are also inherited and fixed for the different lines but the numbers fixed in the different strains have no correlation with leucocyte number or resistance. This is as we might expect if the erythrocytes play no part in the immunity.

X-ray exposure is known to modify the numbers of blood cells. Suitable use of X-rays should thus furnish further information on the part these cells play in the genetic resistance. Some 1256 mice have been treated with X-rays for these experiments; the dose ranged from 0 to 700 e.s.u. per square centimeter of body surface. With more than 700 e.s.u. the mice are so adversely affected as to show severe damage; above 1200 e.s.u. many mice die as a result of the X-ray treatment alone.

The mice were irradiated at about 52 days of age, then allowed a period of 8 days in which to recover from any immediate damage. At about 60 days of age, the S, RI, Z, and E strains were inoculated with 200,000 organisms of medium-virulent typhoid culture. The L and Ba strains were so susceptible that they were inoculated with but 100 organisms of the same culture. The results of this treatment are shown in fig. 6.

All seven mouse strains show a pronounced effect of the previous X-ray treatment on the capacity of the animal to survive inoculated mouse typhoid. The data are plotted as the log of the percentage of surviving mice against the X-ray dosage. Irregularities in individual observations occur but the over-all result is a uniform decline in survival as the X-ray dosage is increased. Between strains there is again a variation in the slope of this decline. This is to be expected on purely random grounds. Some measure of its possible significance can be had by comparing the two S strains as these two curves are really tests for but one strain.

It is evident from the plot that the effect of the X-rays on survival takes the form of the simple exponential equation:

$$\text{Survival} = ae^{bd}$$

Where *a* is a constant, *e*, the base of the natural logarithms, *b*, the term measuring the effectiveness of the X-rays, *d*, dose, measured in e.s.u. The effectiveness of the X-rays, *b*, for the two like tests on the same strain, S, is —.0009 and —.0023. The variations of our experiments are evidently such that a difference of this magnitude can be interpreted as due to uncontrolled causes. The constants for the X-ray effectiveness of all strains or the general slopes of the survival line are:

Strain	*Slope (b)*
S	—0.0009
S	—0.0023
RI	—0.0004
Z	—0.0015
E	—0.0028
L	—0.0007
Ba	—0.0014

It is evident that the slope constants are all within the same range. In fact, tests for significance of the differences show that the error within each strain .036 with 17 degrees of freedom is larger than the differences between regressions .027 with 6 degrees of freedom. We may therefore conclude that X-rays affect all strains in a similar manner.

This is important confirmatory evidence that the leucocytes are significant to the physical basis of genetic resistance to mouse typhoid. It is well known that X-rays destroy leucocytes. If the absorption of one unit of ray energy is sufficient to cause the destruction of a leucocyte or its primodial cell, then we would expect the leucocytes to decline according to the form, leucocytes $= ae^{bd}$, as the X-ray exposure, d, is increased. There is a linear relation between survival to typhoid and numbers of leucocytes. We should therefore expect that this decline would give comparable declines in the survival of the different strains to typhoid as the X-ray dosages increase. The data fit this view.

ORGAN AND CELLULAR DIFFERENTIATION IN DISEASE RESISTANCE

Clinical and cytological observations of mice which succumb to inoculations of 200,000 bacteria have shown that mice of the susceptible strain develop extensive lesions in the spleen and moderate ones in the liver. Mice of the resistant strain show no necrosis of the spleen and extensive destruction of the liver tissues.

Cytological studies of liver and spleen in inoculated animals show that with the onset of morbidity glycogen practically disappears from the livers of mice with low and intermediate resistance while glycogen storage is normal in the most resistant mice. With progress of the disease, susceptible mice show extreme fatty degeneration of the liver while the resistant strains show degeneration only as associated with the lesions. These observations indicate that the tissues of the resistant host are able to carry on their normal function even in the presence of the relatively large amounts of toxin which must be present to produce the severe hepatic necrosis which is characteristic of resistant strains.

Bacteria are visible in the liver and spleen about four days after inoculation. In the susceptible strains, bacteria always appear and usually continue to multiply until they kill the host. In mice of intermediate resistance the bacteria are present in the liver and spleen but in about half of the mice they disappear by the 8th to 11th day. The resistant mice, on the other hand, appear to destroy the bacteria rapidly for bacteria are never visible in the liver and spleen of most animals. When bacteria are present, they are usually found in definite lesions.

The spleens of the different strains appear to differ in the white pulp. Resistant strain shows more of that part of the organ than the susceptible strain. Such a difference should be directly associated with the number of macrophages per unit area of the spleen for these cells evidently arise from the lymphocytes of the white pulp both normally and during the progress of the disease.

The genetic capacity to resist or be susceptible to mouse typhoid evidently depends upon several different types of organ and cellular reaction. The particular

types are fixed within the strain by their genetic constitutions.

It might be thought that the humerol elements in the blood might also vary from strain to strain and play a part in disease resistance. Studies have been made on the agglutinative power and also on the bactericidal power of the serum of different strains.

AGGLUTININS AND DISEASE RESISTANCE

It is conceivable that the genetic selection and controlled breeding of the resistant lines could have led to fixation of natural agglutinins to *Salmonella* within these lines. If this were true, their immunological differences could be accounted for by such differences. Tests for natural agglutinins have been carried out on more than 100 mice of each strain but none have been found. Natural agglutinins seem to play no part in the genetic resistance observed in our strains of mice.

BACTERICIDAL POWER OF THE SERA

The natural bactericidal power of the blood could also have played a part in the genetic resistance. Tests for it in more than 60 mice of each strain have also shown it lacking.

GENERAL VIGOR AND DISEASE RESISTANCE

Since the days of Hippocrates it has been thought that some over-all element of disease resistance as general constitutional well-being played a definite part in resistance or susceptibility to many different diseases. While we have shown that such a general over-all condition does not seem to play any part in the resistance to unrelated diseases, it has seemed worth-while to examine the question for the typhoid organism.

Duration of life appears to be a good measure of vigor. A study was made of the duration of life of our six strains of mice. These studies show great differences in the length of life. Some strains are short-lived, others long-lived. Search for infectious causes of death have failed to reveal any of the common disease agents. At 60 days of age one could not pick out the long-lived strains from the short-lived strains by their appearance. In fact, in ordinary life, where internecine strife is a contributing cause of death between the males, one of the short-lived strains is a constant winner. The ability to survive is a clear-cut inherited difference.

This character has a high correlation with resistance to typhoid. The long-lived strains have high resistance; the short-lived are susceptible. Something in the genetic make-up of these long-lived strains favors resistance to typhoid even though previous contact with the organism has been wanting.

DISCUSSION AND SUMMARY

Before attempting an explanation of these results it may be wise to summarize them. For the pathogen a given organism may gain or lose virulence with equal

suddenness. The gain or loss of virulence may extend to very large populations if sufficient time elapses and the selection pressures are great enough. The changes are entirely comparable to mutations in the phenotype of higher forms. Bacteria or viruses may mutate to a multitude of various different types which later will breed true to the new type. These facts point to a relatively large number of genes, with capacities for variation within the pathogen. Some of these genes may mutate independently of virulence. The mutation of others may change the virulence type of the organism. This, too, would be expected from evidence on *Drosophila;* visible mutations may sometimes affect sterility whereas other mutations have no effect on sterility. There seem to be two rather distinct types of these variant changes. One of these has progeny showing only the variant type; the type is stable to the mutant type in the sense that most mutations in higher forms remain stable. Another type has progeny which show both the variant type and a new type. Progeny of the new type remain stable to the new type. Progeny of the variant continue to break up in successive generations to the variant type and to the stable type. These two types of variants, stable and unstable, occur with fair frequency. Temperature effects on the rate of change of the two types suggest that the mechanism behind the mutation processes may be different for each.

Mutations in tobacco mosaic virus show a pattern similar to that of bacteria. The mutations may or may not affect virulence. The different stable mutants observed from a given parent strain are frequently difficult or impossible to separate serologically from the parent strain. On the other hand, widely different virus strains may show three or four antigenic types. These facts suggest that tobacco mosaic virus, although possibly a single molecule, may have multiple antigenic properties, despite the fact that a mutant may not always be distinguished from its parent type in this respect.

These facts have important bearings on gene structure. They would seem to show that if a gene is likewise of molecular type it could have side chains, one responsible for one set of phenotypes and another for another set, the different sets seemingly affecting quite different processes. Such a model of the gene is not the one commonly drawn from the evidence on other forms. The tobacco mosaic units have a structure which suggests another possibility. The tobacco mosaic unit is seemingly built up of sub units, i. e. 2.2 mμ x 2 mμ x 2 mμ or perhaps more probably 37 mμ x 15 mμ x 15 mμ; the larger molecules being the multiples of this type, i. e. 300 mμ in length. The variant types could each be associated with a different sub unit, the whole being more like a chromosome in structure as Bawden has also suggested. The fact that the nucleic acid in each is of different type does not invalidate this parallelism but rather emphasizes the significance of the two models.

For an examination of how this variant behavior of the pathogen affects the host, we may turn either to the results on the domestic fowl and its typhoid organism, *Shigella gallinarum,* or to the mouse and its typhoid organism, *Salmonella*

typhimurium. The host, through genetic means, may be differentiated into pure-breeding strains each with characteristic resistance to a given line of the pathogen. In the mouse we have six such strains. The Ba and L strains have low resistance, the E and Z strains have medium resistance, and the S and RI strains have great resistance to the disease organism. The resistance differences appear due to the cellular pattern of the mouse as indicated by the blood, spleen, and liver. We have been unable to find evidence for the resistance differences being due to any humerol constituents as, for instance, agglutinins or bactericidins in the blood serum. General constitution as measured by normal duration of life is highly correlated with strain resistance to typhoid and may be a cause of the degree of resistance.

There is an interaction between the genetic constitutions of the pathogen and the host as shown by testing the different strains of mice with the different lines of bacteria. With living organisms the low virulent bacteria show a few deaths in the susceptible mice, a very few in the medium-resistant, and almost none in the resistant strain. With medium-virulent organisms there are a fair number of deaths in the susceptible mice, a lesser number in the medium-resistant mice, and a very few in the resistant mice. With the highly virulent line the deaths in the susceptible group are almost 100 per cent, are medium to high in the medium-resistant, and are low to medium in the resistant mice. If killed bacteria of the three lines are used, the same resistance levels are denoted in the different strains of mice but to get them it is necessary to use 100 times as many or more of killed organisms than of the living organisms. These facts show that the capacity for growth of the bacteria is not the only factor in this disease difference. Rather it indicates that there are different levels of endotoxin in the three lines of bacteria, each working on hosts of differing resistance. The mechanisms of these changes may be considered as follows: The three lines of bacteria may be regarded as of three distinct genotypes. Under the particular gene influence these lines develop an endotoxin within the bacterial cells. The endotoxin is such that it does not escape from the cell into the surrounding medium unless the cell itself is broken down. The endotoxin within each cell could be either different in amount for each line or different in type. As yet we have no evidence on this point. The living bacteria introduced into the host result in increasing amounts of endotoxin with the growth and death of the bacteria. The bacteria generate humerol antibodies, demonstrable through agglutination of the bacteria, in the host. These humerol antibodies probably take little part in the animal's immediate resistance for they appear too late in the course of the disease. They would, however, have the capacity to combine and neutralize a fair amount of toxin at a later date providing the animal survives. The main resistance mechanism so far analyzed appears to be the leucocytes of the blood and macrophages of the spleen and liver. These cells have affinity for the bacteria and for their endotoxins. The rapid disappearance of the bacteria in the resistant animals and the evident resistance shown in the formation of rather extensive lesions in some of the strains support this view.

Immunizing mice with killed bacteria prior to inoculation with living organisms indicates the mechanism by which artificial immunization may take place. In immunization the bacteria with the inheritance for low virulence have a low immunizing value. The bacteria for higher virulence have higher immunizing value. These facts point again to the genes in each of the bacterial lines as governing the formation of endotoxin either differing in amount or kind. On introduction into the host each bacterial line generates a characteristic amount of humerol antibodies in the host circulation. The cellular resistance of the host remains the same as or is increased somewhat over that of the unimmunized mice. When these immunized mice are inoculated with living bacteria of one of the different lines, it takes 100 or more times the number of bacteria to cause death as it would if the mice were unimmunized. The comparative death rates of the different strains of mice, however, remain as they were for the original untreated strains. The introduction of the dead bacteria into the host has made endotoxin characteristic of the particular line of bacteria. These endotoxins have resulted in the generation of humerol antibodies demonstrable through agglutination. These antibodies are of medium strength for the avirulent line of bacteria and of fair strength for the higher virulent lines. Presumably on introduction of the living organism these antibodies combine with them to agglutinate them and possibly to destroy some of the endotoxin through neutralization. The capacity for the resistance is thus increased about 100 fold. The cellular resistance mechanism likewise holds for the strain. Both the strains of mice and the lines of bacteria hold their relative positions in the immunization picture that they held in the case in which no immunization took place. It is simply that the levels of resistance are on a higher plane.

Genes differing possibly in side-chain structures, form H and O antigens and endotoxins differing in their capacities to produce agglutination or death. The antigens or endotoxins may reflect differences either in chemical structure of the genes as different antigens or endotoxins or, what now seems more likely, the capacities of the genes to produce small or large quantities of a single antigen or endotoxin. Host differences are attributable to host gene differences leading to the production of few or many of particular types of cells, i. e. macrophages; with specific capacities to destroy these bacteria or neutralize small or large amounts of endotoxin.

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